TOWARD HEPATITIS B VACCINES*

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ACTIVE immunization against hepatitis B was attempted by Krugman, Giles, and Hammond[1, 2] and Krugman and Giles,[3] using as the immunogen a known infective human serum (MS-2 serum that contains hepatitis B virus and was diluted 1 in 10 and heated at 98° C. for one minute. The serum treated in this manner was not infective and it successfully prevented or modified hepatitis B in 20 (69%) of 29 children who were challenged after four to eight months with the untreated ineffective MS-2 serum. The illness in the remaining nine children was generally mild; jaundice occurred in one volunteer. Abnormal levels of serum glutamic oxaloacetic transaminase were noted in 12 (41%) of 29 children. Hepatitis B antigen was detected in the serum of 12 children and the antigen persisted in three of the 29 children, giving a chronic carrier rate of about 10%, whereas in a control group of 24 nonimmunized children a chronic carrier rate of 37.5% was observed after exposure to MS-2 serum. Hepatitis B antibody, assayed by sensitive techniques, was induced in eight of 29 children, but it was transient in six of the children and, further, one of the children with "vaccine"-induced antibody became a persistent carrier of the antigen after challenge. The results with the heat-inactivated serum were essentially the same after one, two, or three inoculations.

Diluted and heat-treated whole serum may be regarded as an inactivated "vaccine," but it is a crude way of inducing immunity and it is improbable that such material will be licensed for general use.

The repeated failure to passage hepatitis B virus serially in tissue or organ cultures[4] has hampered progress toward the development of a safe

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and effective vaccine. Attention has therefore been directed more recently toward the use of other preparations for active immunization against hepatitis B.

Hepatitis B Virus

The close association between hepatitis B antigen and human hepatitis B virus is now firmly established.[5] The description by Dane, Cameron, and Briggs[6] of distinct virus-like double-shelled 42 nm. spheroidal particles in the serum of some patients with acute illness associated with hepatitis B antigen was followed by the finding by immune electron microscopy of a second antigen-antibody system in this infection.[7] After detergent treatment of pellets of antigen obtained by ultracentrifugation of whole serum, the 42 nm. Dane particles separated into an outer coat which possessed hepatitis B antigen activity and an inner component or core, 27 nm. in diameter, resembling morphologically the enteroviruses. Antibody in convalescent hepatitis B serum reacted with the core to yield immune aggregates resembling those previously seen in homogenates of the liver obtained at autopsy from patients with hepatitis B. The core antibody was found to have an entirely different specificity from antibody to the outer (hepatitis B surface antigen) coat. Hoofnagle, Gerety, and Barker[8] demonstrated that all patients who had infections associated with hepatitis B surface-antigen developed complement-fixing antibody to the core. The titre of the core antibody fell to low levels after recovery from natural hepatitis B infection, but in chronic carriers titres of core antibody remained high. These and other observations suggested that core antibodies are produced in response to replication of the virus. Kaplan and his co-workers[9] demonstrated DNA-dependent DNA polymerase activity in association with the Dane particles and evidence was provided suggesting that the polymerase activity was associated with the cores, released spontaneously or by detergent treatment. The data are consistent, therefore, with the view that the Dane particle is the human hepatitis B virus, the core being the virion nucleocapsid and hepatitis B antigen the outer protein coat.

Krugman and his co-workers[10] recently examined serial serum samples for hepatitis B-specific DNA polymerase and for core antibody. These sera were obtained from volunteers exposed to the MS-2 strain of hepatitis B virus and to heated-inactivated MS-2 serum. Hepatitis B surface antigen appeared first in the serum of infected volunteers, fol-

lowed by DNA polymerase activity then core antibody, before or at the time of elevation of serum transaminase. DNA polymerase activity persisted for days or weeks in acute cases and for months or years in chronic carriers, while core antibody persisted in all cases. Hepatitis B surface antigen, DNA polymerase, and core antibody were not found in persons inoculated with heat-inactivated MS-2 serum. It was concluded that DNA polymerase activity identifies the period of peak replication of hepatitis B virus and that the core antibody reflects recent or continuing replication of the virus.

Hoofnagle[8] reported that titres of core antibody in three patients were not raised by re-exposure to hepatitis B antigen-positive serum. These and other findings suggest that core antibodies are produced in response to replication of the virus in the liver, these antibodies were not raised by re-exposure to serum containing hepatitis B antigen and, unlike antibody to the surface antigen core, antibodies did not correlate with resistance to reinfection nor did they signal recovery from infection.

Subunit Hepatitis B Vaccine

Isolated viral coat protein challenges the body's immune mechanism in the same way as the whole infectious agent and the possibility of using purified hepatitis B surface antigen particles, which are free of nucleic acid and therefore of infectivity, appears attractive. However, such an approach may be precluded by the amounts of host protein that may form complexes with the viral protein in quantities which appear to be far in excess of the protein coat of most recognized viruses. These host proteins may include various pre-existing structures of the liver cell and may thus induce undesirable immunological reactions.[11] Subunits of the antigen, in the form of small polypeptides, on the other hand, offer a much greater promise as possible immunogens.

The close association of hepatitis B surface antigen with normal serum components, confirmed in our laboratory by radioimmunoassay of fractionated material, has been an acknowledged difficulty in the development of purification techniques for separation of the antigen from serum prior to biochemical and serological characterization. This association with other proteins in unfractionated serum was confirmed by isoelectric focusing in a sucrose gradient. The antigen carrying the subdeterminants *ad*+*y*- and morphologically consisting almost entirely of

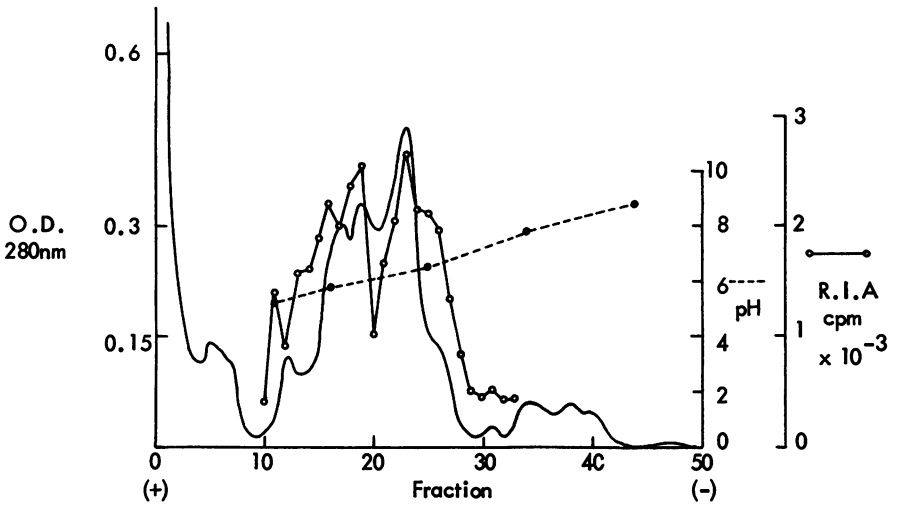

Fig. 1. Isoelectric focusing of serum containing hepatitis B antigen in a 0-60% w/v sucrose gradient containing carrier ampholytes (Ampholine) to establish a *p*H 3-10 gradient at a final concentration of 1%. After 72 hours, fractionation of the separated proteins was followed by radioimmunoassay of each fraction. Only positive results for the hepatitis B surface antigen are shown.

the small 20 nm. particles was found to be associated with serum proteins over a *p*H range of 4.0 to 7.0 (Figure 1). Howard and Zuckerman[12] have previously shown that if the major portion of serum proteins was removed by gel filtration, the technique of electrofocusing separates the antigen from the remaining unwanted serum protein and reveals a heterogeneity in the surface properties of the small antigen particles. The isoelectric points of the two isolated bands were found to differ according to the nature of the subdeterminants and to share at least one common antigenic determinant as demonstrated by immune electron microscopy.[13]

In a further series of experiments, purified hepatitis B antigen was iodinated with the radioisotope iodine^{125} after separation from serum proteins by polyethylene glycol precipitation and equilibrium centrifugation in caesium chloride. The iodinated antigen was found to focus at slightly higher isoelectric points. The two heterogeneous bands were analyzed for their constituent polypeptides by polyacrylamide gel electrophoresis (Figure 2). The profiles obtained were found to bear a close resemblance to gels stained for protein with Coomassie Brilliant

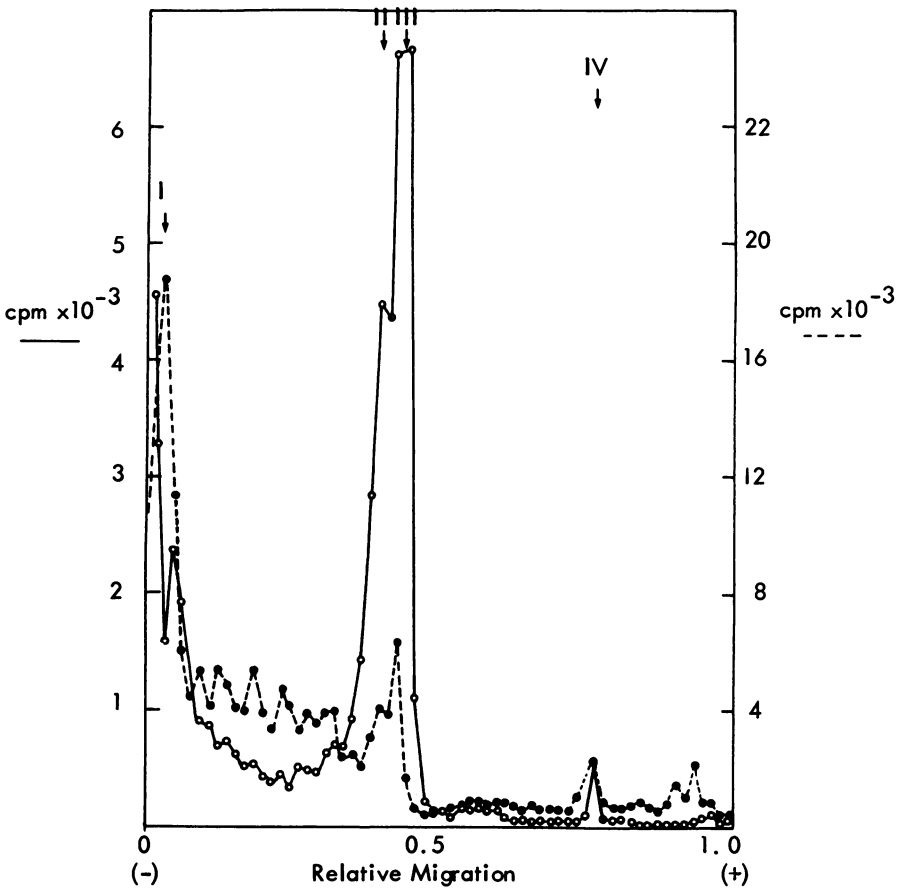

Fig. 2. SDS-acrylamide gel electrophoresis of separated bands of iodinated hepatitis B surface antigen. Antigen of pI 4.7 contains a polypeptide of molecular weight 90,000, whilst antigen of pI 4.9 possesses the slightly smaller major polypeptide component of molecular weight 82,000. Both populations of particles contain the smaller 30,000 molecular weight component, as shown by iodination.

Blue. The average molecular weight of the isolated polypeptides was estimated from a series of experiments according to the method of Summers and Maizel[14] and shown in Figure 3. Each band was found to contain one of the major iodinated polypeptides, together with a smaller polypeptide which had a molecular weight of 30,000. Both of these major peaks of activity are integral components of the antigen as shown by comparison with similarly prepared antigen iodinated by the lactoperoxidase method (Figure 4). With a molecular weight of 87,000,

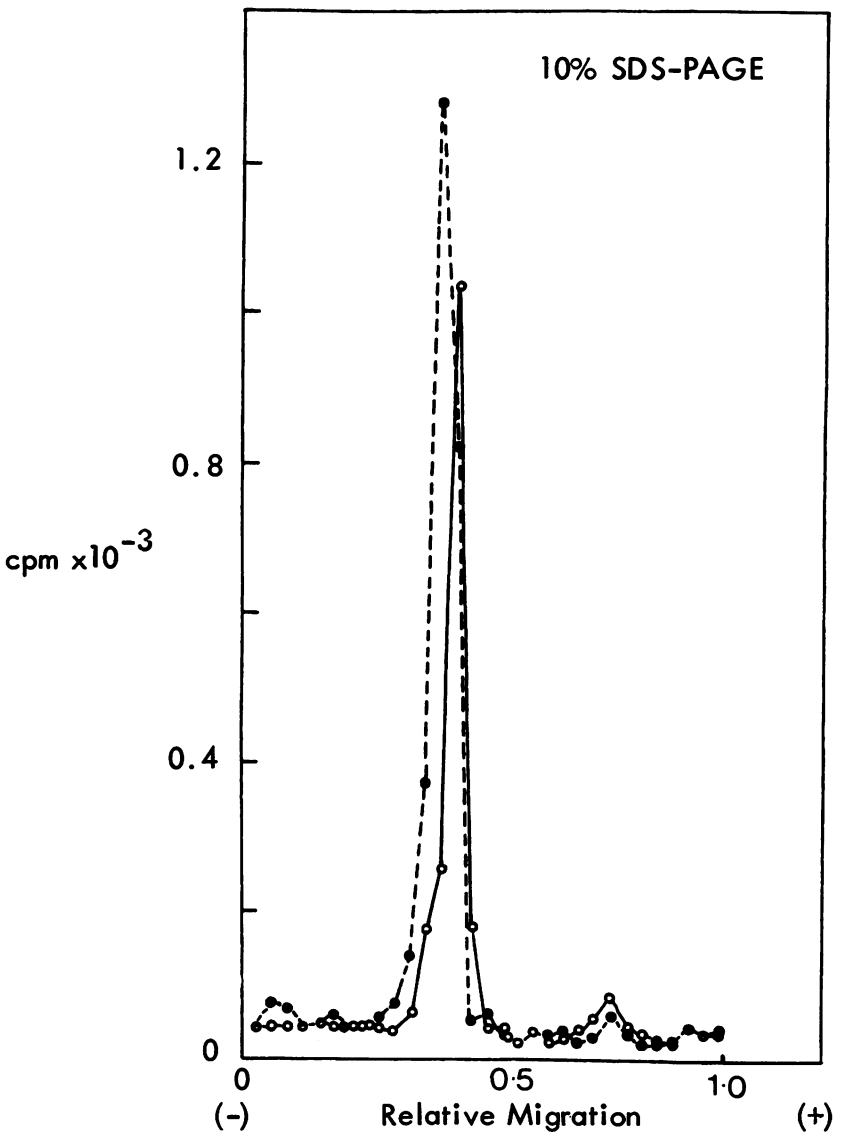

Fig. 3. The molecular weights (x10^4) were obtained for polypeptides II, III, and IV by comparison in a series of experiments with the relative migration of the following: B, bovine serum albumin; O, ovalbumin; C, chymotrypsinogen A; and M, myoglobin. Polypeptides were estimated as being 90,000 (II) 82,000 (III), and 30,000 (IV) in molecular weight.

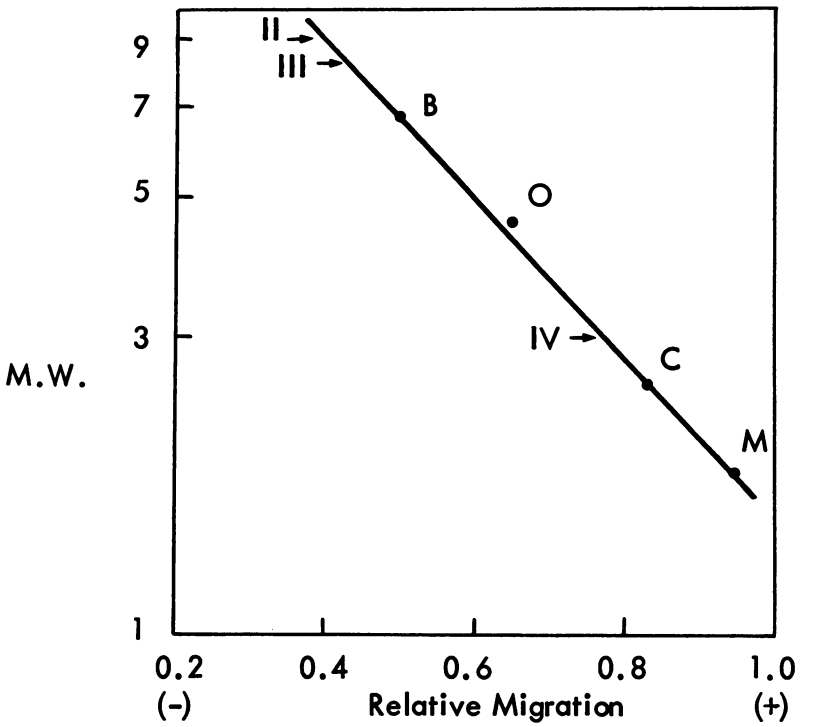

Fig. 4. SDS-acrylamide gel electrophoresis of purified hepatitis B surface antigen (Subtype *ay*) iodinated by the chloramine T (solid line) or the lactoperoxidase (dashed line) methods. Peaks II, III, and IV are believed to be integral protein components of the small 20 nm. diameter particles.

lactoperoxidase specifically labels those components whose tyroxyl residues lie on or close below the surface of the antigen particle.[15] Both of the major iodinated polypeptides were isolated independently by the introduction of ^{14}C into the antigen in a parallel experiment. It was found that antigen trace-labeled with ^{14}C behaved in isoelectric-focusing experiments in a similar way to iodinated hepatitis B antigen. Removal of the lipid component by prior incubation of the sample in the nonionic detergent Nonidet P_{40} and mercaptoethanol before isoelectric focusing in a urea gradient released the two major components from the antigen. Each component possesses a higher unique isoelectric point, compared to the intact original particles. The increase in isoelectric point was paralleled by a significant increase in the sedimentation coefficient of the

radiolabel, presumably as a result of loss of the lipid moiety and the alteration of the secondary structure of the separated polypeptides.

There is disagreement in the estimated number and concomitant molecular weights of isolated polypeptides reported by various workers.[16-19] Prior separation by isoelectric focusing and the procedures for electrophoresis used in our laboratory permitted fine resolution of the constituent polypeptides of hepatitis B antigen. Several estimates contain at least one polypeptide in the molecular weight range of 80,000 to 100,000. A slower migrating component may be resolved into at least two polypeptides after trace-labeling, each being partly responsible for an apparent surface heterogeneity of the small 20 nm. particles of the antigen. Iodination of the antigen in our experiments followed closely the analysis obtained by staining separated polypeptides. The results obtained with the iodinated antigen can be applied to the treatment of unlabeled material used for preparative work.

Such polypeptide preparations should be investigated as potential vaccines for hepatitis B by defining the immunogenic moiety by studies in appropriately selected nonhuman primates such as chimpanzees.

Synthetic Hepatitis B Vaccine

An immunochemical study of purified hepatitis B antigen is essential for the understanding of this unique antigen. Analogous to the TMVP decapeptide, the primary sequence of the haptenic peptide of hepatitis B antigen may provide another approach for a development of a synthetic peptide, which, when coupled to a macromolecular carrier, could serve as a suitable immunogen. Once detailed data are available on the protein, peptide, and amino-acid composition of this antigen, it should be possible to define by animal immunization the moiety responsible for the antigenic activity.

There are reports in the literature to support the feasibility of such an approach. For example, in 1966 Stewart[20] defined the antigenic moiety of TMV protein decapeptide. Arnon[21] showed that it was possible to use a synthetic macromolecule for eliciting antibodies reacting exclusively with a specific region of a native egg-white lysozyme. This was achieved by synthesizing a particular segment of the enzyme from its amino-acid components, attaching the peptide to a synthetic polypeptide carrier, and using the conjugate for immunization. The resulting antibodies reacted with native lysozyme in a unique, conformation-

dependent antigenic determinant. Of course, in the case of lysozyme both the amino-acid sequence and the three-dimensional structure are known, and the synthesized peptide was designed on the basis of previous information concerning its contribution to the antigenic specificity of the molecule.

A similar approach might be attempted for type B hepatitis, depending largely on the success in elucidating the structure of hepatitis B antigen, since there is little doubt that polypeptides and other moieties such as specific lipoproteins can be attached to a macromolecular carrier[22] for subsequent immunization. The prospects of active immunization against hepatitis B appear brighter.

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